

**Title: Dimethylaminoethyl Methacrylate Copolymer-siRNA nanoparticles for silencing therapeutically relevant gene in macrophages**

Ratnesh Jain\*<sup>1,2</sup>, Prajakta Dandekar\*<sup>1,2</sup>, Brigitta Loretz<sup>2</sup>, Marcus Koch<sup>3</sup>, Claus-Michael Lehr<sup>2</sup>

<sup>1</sup>Institute of Chemical Technology

NP Marg, Matunga

Mumbai 400019 India

<sup>2</sup>Department of Drug Delivery (DDEL),

Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS),

Campus A4 1, Saarland University, Saarbrücken, 66123 Germany

<sup>3</sup>Innovative Electron Microscopy, INM – Leibniz Institute for New Materials, Service Group

Physical Analysis, Campus D2 2, Saarland University, Saarbrücken, 66123 Germany

\*Corresponding Authors

<sup>1</sup>Institute of Chemical Technology

NP Marg, Matunga

Mumbai 400019 India

Tel: +91-22-3361-2029

Fax: +91-22-3361-1020

Email: rd.jain@ictmumbai.edu.in

## Supporting Information

### S1. Complexation efficiency

DMC-pDNA/siRNA complexes were prepared at different w/w ratios from 1 to 10 and the complexes, along with 6× loading buffer, were loaded onto 0.5 % agarose gels containing 1% ethidium bromide for visualization of the bands. The gel was run in 0.5× Tris/Borate/EDTA (TBE) buffer at 50V for 60 minutes. Unbound siRNA was maintained as a control during the study. siRNA bands were visualized using an Peqlab Fusion 7 (PEQLAB Biotechnologie GMBH, Erlangen, Germany).

### S2. MTT Assay

This assay is based on the capability of the mitochondrial dehydrogenases of the living cells to reduce MTT, a yellow tetrazolium salt, to purple formazan crystals. It is one of the most extensively employed assays of cellular death which depends on the toxicity of test material. Cytotoxicity assay is particularly important for DMC nanoparticles due to the possibility of cellular accumulation, retention and non-biodegradability of the polymer.

Prior to the assay, Murine macrophage cell line, RAW 264.7 cells were seeded in 96-well plates at a density of 20,000 cells/well. Once confluent, the cells were incubated with unloaded nanoparticle at different concentrations (75-200 µg/ml) for 24 h. During the assay, cells grown in culture medium only were considered as high control (100% cell viability) and others incubated with Triton X-100 (1%, w/v) were used as low control (0% cell viability). After incubation with nanoparticles, the cells were washed with PBS after taking out the nanoparticles. Thereafter, MTT solution (5 mg/ml in PBS pH 7.4) was added along with normal medium (1:10; v/v) followed by incubation for further 4 h. The precipitated formazan was then dissolved using DMSO (200 µl) and quantified by measuring the absorbance at 550 nm in a multiwell plate reader (Tecan Deutschland GmbH, Crailsheim, Germany). Samples were applied in quadruplicates. Cell viability (%) was calculated by the following equation:

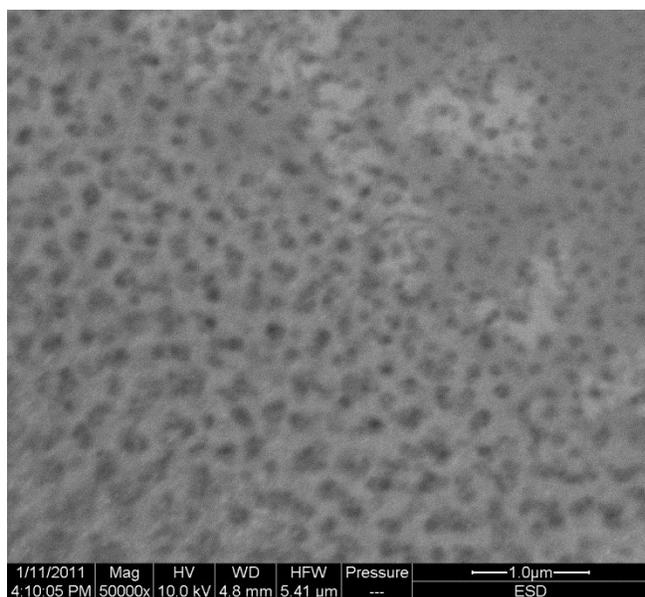
$$\% \text{ Cell Viability} = \frac{Abs_{exp}^{550} - Abs_{low\ control}^{550}}{Abs_{high\ control}^{550} - Abs_{low\ control}^{550}} \times 100$$

Means and relative standard deviations (RSD) were calculated.

### S3. CLSM Staining

The cells were washed twice with PBS and cell membranes were stained with 25  $\mu\text{g}/\text{ml}$  of the FITC-WGA (Emission: 515 nm, Excitation: 495 nm) ; further, these cells were incubated for 10 min in the incubator. Later, cells were again twice washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cell nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, emission: 461 nm, excitation: 374 nm; Fluka Chemie GmbH, Buchs, Switzerland) and cells were incubate for 10 min at room temperature. Afterwards cell were washed again with PBS and stored in 500 $\mu\text{l}$ /well PBS till further analysis at 4°C.

### S4. ESEM



*Fig. S1 A. Liquid film data of DMC Nanoparticles*

### S5. Cell Uptake Pathways

Cells were incubated with the inhibitors for 3 h and then with DMC nanoparticle along with inhibitors, for another 4 h. Nanoparticle were removed after 4 h and the cells were washed twice

with PBS. The cells were then incubated with complete medium containing inhibitors for further 18 h. After this total duration of 24 h, the cells were fixed and stained. The cells were stored in 500µl of PBS /well, at 4°C, till further analysis. The imaging was performed as described confocal microscopy section of main manuscript.

**Table S1. Endocytic inhibitors used to investigate the mechanism of uptake of nanoplexes**

<b>Endocytic Inhibitor</b>	<b>Function</b>	<b>Dose</b>	<b>Experimental protocol</b>
Chlorpromazine	Clathrin-dependent pathway	10 µg mL <sup>-1</sup>	3 h Inhibitor+cell 4 h Inhibitor+Cell+Nanoparticles 17 h Inhibitor+Cell
Nystatin	Caveolae-dependent pathway	10 µg mL <sup>-1</sup>	3 h Inhibitor+cell 4 h Inhibitor+Cell+Nanoparticles 17 h Inhibitor+Cell
5-(N-Ethyl-N-isopropyl)amiloride	Macropinocytosis, Phagocytosis	5 µg mL <sup>-1</sup>	3 h Inhibitor+cell 4 h Inhibitor+Cell+Nanoparticles 17 h Inhibitor+Cell

Quantification of nanoparticles within the cell and outside the cell is a matter of debate and so far no validated methods have been reported, which justify and provide clarity about the exact number of nanoparticles present within the cell. The cells were initially counted manually for the number of cells positive for the presence of nanoparticles, by manually counting the number of fluorescent spots per field in confocal image. The nanoparticles (red spots) located inside the cells (Fig. S2) were counted and the red spots outside the cells or on the membrane were excluded in this study. Z-stacks of these images were analyzed for accuracy, wherever necessary. The results indicate (Fig. S2A) that when incubated with CHL,  $73.96 \pm 13\%$  of the counted cells exhibited internalization of the nanoparticles, in case of NYS this value was  $70.33 \pm 9\%$ , while for AML just  $8.2 \pm 0.4\%$  of the cells exhibited internalization of nanoparticles. The visual counting clearly supported the CLSM observations and suggested the uptake of DMC nanoparticles by macropinocytosis/phagocytosis. However, for further confirmation, and to avoid human error in manual counting, these observations were confirmed by analyzing the weighed number of nanoplexes in CLSM images of cells. The quantification of nanoparticles was performed using multiphoton-pixel analysis method. This method is based on pixel analysis instead of analyzing the particle intensity. The pixel intensity provides information about the weight number of nanoparticles in the region of interest. The results have been presented in Fig. S2B.

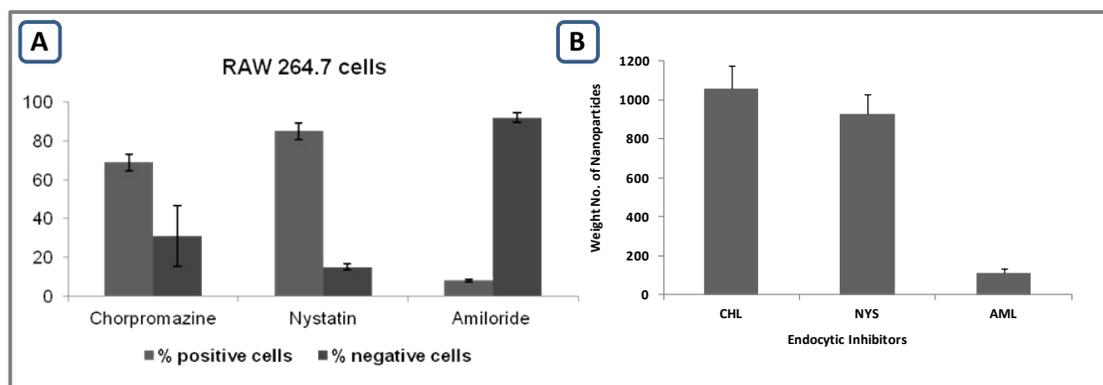


Fig. S2 A. Quantification of nanoparticles by manual counting of positive and negative cells. B. Quantitative analysis of nanoparticles by multiphoton-pixel analysis method.

### S6. Transient Gene Expression: Luciferase

RAW 264.7 cells were grown in 24 well plates 24 h before the luciferase gene was introduced by transfection using pGL3-control plasmid (1  $\mu\text{g}/\text{well}$ ) and jetPRIME™, according to manufacturer's instructions. Following 3 h of transfection period, the medium was removed and the cells were washed with PBS twice. Thereafter, the cells were incubated with 500  $\mu\text{l}$  of fresh medium, without serum, containing 100  $\mu\text{l}$  of DMC/siRNA nanoparticles, naked siRNA, siRNA-jetPRIME complexes for duration of 4 h. The concentration of siRNA in each case was 80 pmol of siRNA/well. Subsequently, the medium containing siRNA, in condensed or free State, was replaced by complete medium and the cells were incubated till a total of 72 h, with medium replacement every 24 h. After 72 h, luciferase activities were determined using Promega Luciferase Assay kit (Promega, WI, USA). The resulting luminescence was analyzed by Tecan microplate reader (Tecan Deutschland GmbH, Crailsheim, Germany). The knockdown efficiency of siRNA was also determined by western blot and Real-Time Polymerase Chain Reaction (RT-PCR).

## Western Blot

Transfected cells (experimental details like 2.9.1) were lysed with 100  $\mu$ L of Cell Culture Lysis Reagent (Promega, WI, USA) / well and the cell lysates were collected as per the manufacturers suggestions. Total protein concentration in the cell lysate was determined by BCA protein assay kit (Bicinchoninic Acid Kit for Protein Determination, Sigma). Whenever necessary, the cell lysate was stored at  $-80^{\circ}\text{C}$ , till further analysis. Western blot was conducted as per a previously published protocol from our research group [19]. The cell lysate, equivalent to 1 mg/mL of protein, was mixed with equal volume of sample buffer (2x) and denatured for 5 min at  $95^{\circ}\text{C}$ . Subsequently, 30  $\mu$ l of each sample was applied to a Polyacrylamid gel electrophoresis (PAGE with 4% stacking gel and 15% separation gel). A prestained protein marker (Spectra Multicolor, Broad Range Protein Ladder, Fermentas) was used as standard. The electrophoresis run for 110 min at 100 V in a BioRad MiniProtean II.

The proteins were transferred from the gel to nitrocellulose membranes (Protran BA 85 Nitrocellulose, Whatman) by electro-blotting (BioRad Mini Trans-blot Cell) at 300 mA for 90 min.

The membranes were blocked for 2 h in blocking buffer and then they were incubated overnight with Luciferase mouse monoclonal IgG<sub>1</sub> (Luci17: sc-57604; Santa Cruz Biotechnology, CA, USA) at a dilution of 1:200 in blocking buffer (according to manufacturer's suggestions), with gentle shaking. The blots were washed three times with TBS buffer for 10 min prior to incubation with chicken polyclonal secondary antibody to Mouse IgG- H & L (HRP) (ab6814; Abcam, MA, USA) at a dilution of 1:3000 in blocking buffer. After washing three times for 10 min with TBS buffer, the blots were developed using Amersham™ ECL™ Prime Western

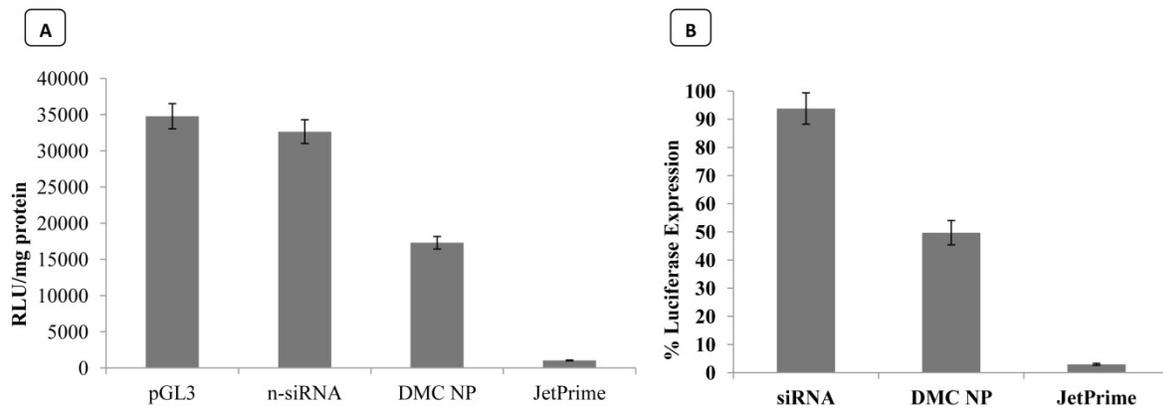
blotting chemiluminiscence kit (GE Healthcare Europe GmbH, Freiburg, Germany) as per manufacturer's protocol. Finally, the blots were analyzed in chemoluminescence mode on Peqlab Fusion 7 (PEQLAB Biotechnologie GMBH, Erlangen, Germany).

## **RT-PCR**

RT-PCR was also conducted as per a previously published protocol from our research group [20]. The cells were transected in exactly the same manner as described before. Thereafter, cells of each treatment group were subjected to RNA extraction using Qiagen RNA isolation kit (QIAGEN - RNeasy Mini Kit, Qiagen, Germany). 1 µg of this mRNA was then used for the synthesis of double stranded cDNA, using the Qiagen QuantiTect Reverse Transcriptase Kit (Qiagen Inc., CA, USA), which was then analyzed by RT-PCR on a Biorad CFX96 real time rotary analyzer (Bio-Rad Laboratories GmbH, Munich, Germany). The cycling protocol employed included an initial denaturation of 1 min at 95 °C and 39 cycles of 95 °C for 10 s, 65 °C for 30 s and 72 °C for 30 s, a final amplification at 65 °C for 1 min, followed by a melt curve analysis (from 60°C to 98°C in 0.5°C steps for 5 s). DyNAmo™ Flash SYBR® Green qPCR Kit (Finnzymes, Fisher Scientific GmbH, Schwerte, Germany) was employed during the experiment. The final reaction mixture contained: 1 µg template DNA , 10 µM primers (1 µL each), 10µl 2x DyNAmo Flash SYBR Green Master Mix in 20 µL total volume . β-actin was included as an internal standard in the experiment and non-template controls, containing water instead of cDNA, were used to rule out DNA contamination of the reagents. Analysis was performed using CFX Manager™ software. Specific primers used were; β-actin: Primer sense sequence 5'-TGC GTG ACA TTA AGG AGA AG-3', Primer antisense sequence 5'-GTC AGG CAG CTC GTA GCT CT-3'; Luciferase (Eurofins MWG GmbH, Ebersberg, Germany): Primer sense sequence

5'-TGG GCT CAC TGA GAC TAC ATC A-3', Primer antisense sequence 5'-CGC GCC CGG TTT ATC ATC-3'.

The results of luciferase knockdown, as analysed by the luciferase assay kit, have been depicted in fig. S3. As evident from the figure, DMC nanoplexes were able to knockdown luciferase expression by 50%, as compared to the cell control transfected only with pGL3. Naked siRNA, however, exhibited only 6% knockdown, which may be attributed to the siRNA degradation before reaching the target site at cytoplasm.



*Fig.S3. DMC nanoplex mediated luciferase gene knockdown A. pGL3 was used to induce luciferase gene expression in RAW 264.7 cell lines. B. Knockdown efficacy of naked siRNA, DMC nanoplexes and commercial transfection reagent.*

The results were further confirmed by western blot and RT-PCR (Fig. S4A and S4B). The protein expression in cells treated with nanoplexes was less as compared to those treated with naked siRNA, when compared with the protein expression in only pGL3 treated cells. Similarly, a significant reduction was observed in mRNA levels in cells treated with DMC nanoplexes, as compared to naked siRNA. Thus DMC nanoplexes exhibited an efficient siRNA delivery and knockdown of reporter gene in RAW 264.7 cells. These results supported our hypothesis and provided the proof of biological efficiency for further evaluation of DMC nanoplexes in silencing a functional gene.

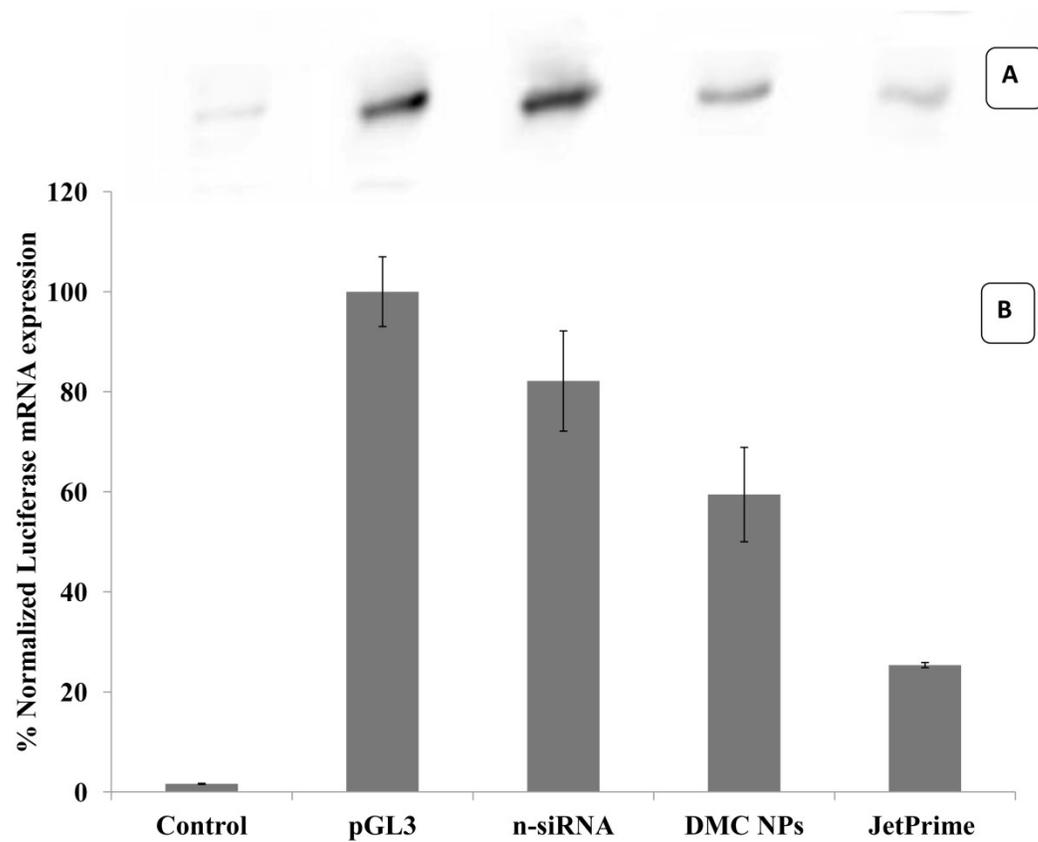


Fig. S4 A. Western blot analysis of luciferase protein expression after transfection of RAW 264.7 cells with DMC (Eudragit or E-100) nanoplex formulated using anti-Luc siRNA (100 pmol) for 72 h. B. Luciferase gene silencing determined using RT-PCR, Luciferase mRNA levels were normalized to  $\beta$ -actin mRNA expression.